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Surfactant Releases Internal Calcium Stores in Neutrophils by G Protein-Mediated Pathway

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ABSTRACT

Pulmonary surfactant with surfactant-associated proteins (PS+SAP) decreases pulmonary inflammation by suppression of neutrophil activation. We have observed that PS+SAP inserts channels into artificial membranes, depolarizes neutrophils, decreases calcium influx following stimulation, and depresses neutrophil functions in vitro. We hypothesize that PS+SAP suppresses neutrophil activation by insertion of cation channels into plasma membrane, depolarization of neutrophils, and G protein-dependent release of Ca^{++} stores, and that gramicidin – a monovalent, cation channel protein – mimics these effects. Human neutrophils were monitored for $[\text{Ca}^{++}]$ responses after exposure to gramicidin alone, gramicidin reconstituted with phospholipid (PLG), one of two different PS+SAP preparations, or a PS-SAP preparation. $[\text{Ca}^{++}]$ responses were reexamined following preexposure to G protein or internal Ca^{++} release inhibitors. We observed that: 1) 1% PS+SAP — but not PS-SAP — causes transient increases of neutrophil $[\text{Ca}^{++}]$ within seconds of exposure; 2) 1% PLG — but not gramicidin alone — closely mimics the effect of PS+SAP upon Ca^{++} response; 3) PS+SAP, gramicidin alone and PLG equally depolarizes neutrophils despite differences among neutrophil Ca^{++} responses; 4) direct inhibition of internal Ca^{++} store release or G protein activation suppresses Ca^{++} responses to PS+SAP and PLG; and 5) preexposure to either PS+SAP or PLG inhibits Ca^{++} influx following fMLP stimulation. We conclude that PS+SAP independently depolarizes neutrophils, releases Ca^{++} from internal stores by a G protein-mediated pathway, and alters subsequent neutrophil response to physiologic stimulants. The mimicking of these results by PLG supports the hypothesis that PS+SAP initiates depolarization via channel insertion into neutrophil plasma membrane.

Key words: Surfactant, neutrophils, calcium, gramicidin, G proteins

ABBREVIATIONS

2-APB 2-aminoethoxydiphenylborate

AUC area under the curve

[Ca⁺⁺] ionized calcium concentration

fMLP formyl peptide

HBSS Hanks balanced salt solution

PS pulmonary surfactant

PL purified surfactant-like phospholipids

PLG gramicidin reconstituted in purified surfactant-like phospholipids

SAP surfactant-associated protein

SKF-96365 1-[β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCl

INTRODUCTION

Pulmonary surfactant, a complex mixture of lipids and proteins, is widely employed for the treatment of respiratory distress syndrome in preterm infants. Natural pulmonary surfactant contains four apoproteins (SAP-A, SAP-B, SAP-C, and SAP-D) in addition to phospholipids and other neutral lipids (1). For the clinical administration of exogenous surfactant to humans, surfactant derived from bovine or porcine sources retains only the hydrophobic apoproteins, SAP-B and SAP-C (PS+SAP), and most synthetic surfactants lack all surfactant-associated proteins (PS-SAP) (2). By meta-analysis of clinical trials evaluating the efficacy of PS+SAP and PS-SAP surfactant compounds, preterm infants treated with PS+SAP require lower mean airway pressures, develop fewer pulmonary air leaks, assume lower oxygen requirements and experience better survival rates than infants treated with PS-SAP (2,3).

The release of proteases and production of oxygen radicals by pulmonary neutrophils is believed to be a key injurious mechanism in development both of chronic lung disease following neonatal respiratory distress syndrome and of acute respiratory distress syndrome in older children and adults (4). In vitro studies examining the effects of PS+SAP on normal human neutrophils have demonstrated decreased neutrophil adherence, aggregation, chemotaxis, respiratory burst, and elastase production following physiologic stimulation (5,6,7,8,9). By contrast, exposure to PS-SAP does not significantly affect neutrophil responses. These studies, when viewed in light of the clinical benefits of PS+SAP over PS-SAP, suggest an anti-inflammatory role for the surfactant apoproteins SAP-B and SAP-C.

We have observed that PS+SAP, as well as, SAP-B and SAP-C isolated from PS+SAP, insert monovalent cation channels into artificial black membranes (10). More recent experiments by our laboratory have demonstrated that PS+SAP, but not PS-SAP, depolarizes neutrophil cell membranes and blocks Ca^{++} influx into neutrophils following physiologic activation (9). Additionally, we have observed that gramicidin-D, a monovalent, cation channel-

forming peptide, depolarizes neutrophil cell membranes and blocks Ca^{++} influx following physiologic activation similar to PS+SAP (9). We hypothesize that PS+SAP inserts monovalent cation channels into neutrophils causing cell membrane depolarization, release and depletion of internal $[\text{Ca}^{++}]$ stores via G protein activation, and decreased neutrophil response to physiologic stimuli. The similarities between PS+SAP and gramicidin regarding observed cation channel insertion and neutrophil depolarization suggest that PS+SAP effects on neutrophils should be closely mimicked by gramicidin. To test these hypotheses, the objectives of the current study are to: 1) determine if PS+SAP induces a rise in cytosolic $[\text{Ca}^{++}]$ dependent upon membrane polarization; 2) ascertain if this effect is mimicked by gramicidin; and 3) test whether cytosolic $[\text{Ca}^{++}]$ rise depends upon release of internal Ca^{++} stores and activation of G proteins.

MATERIALS AND METHODS

Reagents and chemicals. Phosphatidic acid, phosphatidylglycerol, dipalmitoyl-phosphatidylcholine, EGTA, dextran, Ficoll, formyl peptide (fMLP), N-methylglucamine and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, MO). Fura2-AM, fura2 and gramicidin-D were purchased from Molecular Probes (Eugene, OR). Hanks balanced salt solution with (HBSSw) or without Ca^{++} and Mg^{++} (HBSSw/o) was purchased from Bio-Whittaker (Walkersville, MD), Hypaque 76 from Sanofi Winthrop Pharmaceuticals (New York, NY), and heparin from Elfing-Sinn (Cherry-Hill, N.J.). 2-APB, SKF-96365 and pertussis toxin were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). PS+SAP preparations, Survanta[®] and Infasurf[®], were obtained from Ross Products Division, Abbott Labs (Columbus, OH) and Forest Pharmaceuticals (St. Louis, MO), respectively. PS-SAP preparation, Exosurf[®], was obtained from Glaxo-Wellcome (Research Triangle Park, NC).

Purification of neutrophils. Heparinized, venous blood samples were obtained from healthy, adult, human volunteers and separated by Hypaque-Ficoll step-gradient centrifugation, dextran sedimentation and hypotonic lysis as previously described (9,11). Cell preparations typically provided >95% neutrophils confirmed by modified Wright-Giemsa staining, and they were used within four hours of purification.

Reconstitution of gramicidin in phospholipid (PLG). Dipalmitoylphosphatidylcholine dissolved in chloroform was combined with phosphatidylglycerol dissolved in methanol and dry phosphatidic acid providing a final phospholipid molar ratio of 7:2:1, respectively. This phospholipid mixture (PL) was employed for experiments examining the effects of PS+SAP-like phospholipids on neutrophil $[\text{Ca}^{++}]$ changes (12). For experiments requiring gramicidin reconstituted with PL (PLG), gramicidin-D dissolved in methanol was added in a 1:5 (gramicidin:phospholipid) molar ratio to the phospholipid mixture. Chloroform and methanol were removed under N_2 at 45°C. A 1% emulsion of the PLG complex was prepared with 145

mM NaCl by ultrasonication. To change conformation of gramicidin within the phospholipid mixture from a non-conductive to conductive conformer (13), PLG was heated (60° C, 12 hours) following initial preparation, and reheated (60° C, 1 hour) and cooled (24° C, 30 minutes) prior to each experiment. Between experiments PLG was stored at 4-8° C. The estimated final [gramicidin] in experiments employing 1% PLG was 10 μ M.

Cytosolic calcium concentration ([Ca⁺⁺]) measurements. Purified neutrophils were suspended in HBSSw/o and exposed to 2 μ M fura2-AM — the methyl ester form of fura2, a dual wavelength, fluorescent Ca⁺⁺ probe. After incubation (37°C in the dark, 5% CO₂, 45 minutes), cells were sedimented and washed twice in HBSSw/o to remove non-incorporated, extracellular fura2-AM. Neutrophils were resuspended at a concentration of 10x10⁶ cells/ml in HBSSw or HBSSw/o depending upon the experiment. Subsequently, cells (5x10⁶ per cuvette) were examined for fluorescence in an LS50B spectrofluorometer (Perkin-Elmer-Cetus, Norwalk, CT) at excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm. Maximum (R_{max}) and minimum (R_{min}) fura2 fluorescence ratios were calculated by adding 0.1% Triton and 20 mM EGTA, respectively, to cells in the spectrofluorometer. Neutrophil cytosolic [Ca⁺⁺] values were then calculated from the equation:

$$[Ca^{++}] = K_d[(R - R_{min})/(R_{max} - R)]\beta$$

where R is the ratio of the 340 nm/380 nm fluorescence, R_{min} is the minimal ratio of 340 nm/380 nm fluorescence, R_{max} is the maximal ratio of 340 nm/380 nm fluorescence, β is the ratio of 380 nm fluorescence under Ca⁺⁺-free/Ca⁺⁺-saturated conditions, and K_d is the dissociation constant for Ca⁺⁺ binding to fura2 based on calibration curves from our laboratory (9,14).

Early cytosolic [Ca⁺⁺] responses in unstimulated neutrophils were examined following addition of either: 1) 1% PS+SAP; 2) 1% PS-SAP; 3) 1% PL; 4) 10 μ M gramicidin-D; or 5) 1% PLG.

Experiments were performed in both calcium-supplemented (HBSSw) and calcium-free buffers.

Calcium-free buffer was prepared by adding 30 μ M EGTA to HBSSw/o. For experiments

examining Na^+ -dependence of the PS+SAP response, N-methylglucamine (150 mM) was substituted for NaCl in the buffer. Daily pH values of all balanced salt solutions were monitored and maintained at 7.40 ± 0.05 .

For experiments examining the effect of neutrophil membrane depolarization on PS+SAP-dependent cytosolic $[\text{Ca}^{++}]$ peaking, neutrophils were depolarized through incubation with KCl or gramicidin-D prior to PS+SAP exposure (9). Specifically, neutrophils (5×10^6) were exposed (4 minutes, 24°C) to KCl (150 mM) or gramicidin-D ($90 \mu\text{M}$), washed, and resuspended in Ca^{++} -free buffer. Fura2-associated fluorescence changes following addition of 1% PS+SAP were measured as described above.

For experiments testing the contribution of intracellular Ca^{++} stores to $[\text{Ca}^{++}]$ rise, SKF-96365 ($10 \mu\text{M}$) or 2-APB (30 or $100 \mu\text{M}$) was added to neutrophils suspended in HBSS with Ca^{++} 4 minutes prior to addition of PS+SAP, PLG, or fMLP (15). For those experiments testing coupled, G protein activation, neutrophils suspended in HBSS with Ca^{++} (5×10^6 cells/mL) were incubated with or without pertussis toxin (50 ng/mL) (90 minutes, darkness, 37°C , 5% CO_2) (16). Neutrophils were subsequently washed twice with HBSS prior to loading with fura2-AM, resuspending in HBSS with Ca^{++} , and exposing to PS+SAP, PLG, or fMLP .

To examine sensitivity of Ca^{++} influx into stimulated neutrophils following PS+SAP or PLG exposure, 5×10^6 neutrophils were exposed to 1% PS+SAP, 1% PLG or control buffer (HBSSw/o), washed and resuspended in calcium-free buffer. Following stabilization of baseline fluorescence, $1 \mu\text{M}$ fMLP was added and fluorescence monitored. Upon achieving the maximal fMLP -induced $[\text{Ca}^{++}]$ response, 5 mM CaCl_2 was added and fluorescence monitored for an additional 120 seconds. Ca^{++} influx following fMLP stimulation was quantitated by the area under the curve following addition of CaCl_2 and expressed as $\text{nM} \cdot \text{min}$ (9). To provide a baseline control in separate experiments, $40 \mu\text{M}$ EGTA was added following fMLP stimulation.

Membrane Potential Measurement. Neutrophils (1×10^6) suspended in 100 μ l HBSSw were exposed (24°C, 4 min) to either 1% PLG, 1% PS+SAP, 20 μ M gramicidin-D, or HBSSw (control). Cells were washed and resuspended in HBSSw. Potentiometric fluorescent dye diOC₅(3) (225 nM) in HBSSw was incubated (25°C, 4 min) in the dark before baseline fluorescence was recorded at excitation and emission wavelengths of 460 nm and 507 nm (9). Neutrophils were added creating a 20-fold dilution, and upon restabilization of the fluorescence signal, 150 mM KCl was added to fully depolarize neutrophils and quench fluorescence. Quenching was followed until a final plateau was observed. Change in membrane potential was measured as a ratio of the change in fluorescence units (9).

Statistical Analysis. Data were analyzed by paired sample t-tests. P values less than 0.05 were considered significant. All data were expressed as mean \pm SE. *SPSS 7.5 for Windows* software employing the Marquardt-Levenberg algorithm was employed to determine the logarithmic regression of [Ca⁺⁺] peaking upon PS+SAP or PLG concentration. Where dose relationships were sought, correlation coefficients (R^2) were determined by the same software. Replicate experiments used neutrophils from different human donors.

RESULTS

PS+SAP induces a transient neutrophil $[Ca^{++}]$ response. Exposure of neutrophils to 1% PS+SAP rapidly induced transient peaking of cytosolic $[Ca^{++}]$ (Figure 1). Within 60 seconds of PS+SAP addition (closed circles), $[Ca^{++}]$ peaked and was followed by prompt return toward baseline over the next 120 seconds. The observation of this internal $[Ca^{++}]$ response in the absence of extracellular Ca^{++} strongly suggested that intracellular Ca^{++} stores provide the source for cytosolic $[Ca^{++}]$ peaking.

In contrast to the effect of PS+SAP, exposure to 1% PS-SAP (open circles) induced minimal change in neutrophil $[Ca^{++}]$ (Figure 1). Likewise, exposure of neutrophils to 1% PL (composed of phospholipids more closely resembling the phospholipid content of PS+SAP than those of PS-SAP) failed to increase neutrophil $[Ca^{++}]$ (closed triangles). The negligible Ca^{++} response to PS-SAP or PL implied that SAP (SAP-B and/or -C) is necessary for the observed neutrophil Ca^{++} response.

To provide further support for a causal relationship between PS+SAP and the Ca^{++} response by neutrophils, dose effect of PS+SAP on magnitude of intracellular neutrophil Ca^{++} response was examined. At concentration as low as 0.2%, PS+SAP induced release of cytosolic Ca^{++} in Ca^{++} -free buffer (data not shown). Release was not observed at lower concentrations, and concentrations greater than 1% could not be examined because of excessive optical interference. However, between 0.1 and 1% concentrations dose dependency was observed by a logarithmic response of neutrophil $[Ca^{++}]$ to $[PS+SAP]$ (magnitude of Ca^{++} peak = $53.07 + 20.64 \{ \ln[PS+SAP] \}$, $R^2 = 0.93$).

$[Ca^{++}]$ response depends upon membrane polarization and extracellular Na^+ . We hypothesize that the effect of PS+SAP upon neutrophil $[Ca^{++}]$ is mediated by insertion of monovalent cation channels into plasma membrane, and that resulting dissipation of transmembrane, monovalent, cation (i.e. Na^+ and K^+) gradients causes membrane

depolarization via collapse of electrochemical potential gradients. To test for the dependency of Ca^{++} response upon preexisting membrane polarization, we prevented PS+SAP-induced membrane depolarization by depolarizing neutrophils prior to PS+SAP exposure. Two methods were employed to induce membrane depolarization (Figure 2A). The first substituted extracellular 150 mM KCl (open circles) for extracellular NaCl (closed circles), and the second employed 90 μM gramicidin (closed triangles) — a well-characterized, monovalent, cation channel protein causing cell depolarization by the same mechanism proposed for PS+SAP apoproteins SAP-B and SAP-C.

Collectively, both approaches demonstrated that depolarization of neutrophils prior to PS+SAP exposure blocks Ca^{++} response to PS+SAP (Figure 2A). Unexpectedly though, gramicidin achieved this inhibition of PS+SAP-induced Ca^{++} peaking while inducing significantly higher baseline cytosolic $[\text{Ca}^{++}]$ [Control (closed circles): 50 ± 2 nM; KCl (open circles): 52 ± 3 nM; gramicidin (closed triangles): 76 ± 13 nM; $p < 0.05$ gramicidin vs control or KCl].

To test whether hypothesized channels inserted by PS+SAP specifically conduct small monovalent cations, 150 mM N-methylglucamine was substituted for extracellular NaCl. In the absence of extracellular Na^+ , PS+SAP would not be expected to depolarize membrane by conducting Na^+ across plasma membrane. By these experiments, N-methylglucamine (open circles) fully inhibited PS+SAP-induced Ca^{++} peaking without significantly raising baseline cytosolic $[\text{Ca}^{++}]$ (Figure 2B).

Gramicidin mimics PS+SAP effects of on neutrophil $[\text{Ca}^{++}]$ as PLG. Recalling that gramicidin blocked PS+SAP-induced Ca^{++} peaking (Figure 2A) and that this inhibition was achieved by insertion of monovalent cation channels into neutrophil membrane, we reasoned that substitution of gramicidin for PS+SAP should mimic results observed with PS+SAP. Experiments were repeated in both the presence (Figure 3A) and absence (Figure 3B) of extracellular Ca^{++} and while employing a second PS+SAP preparation (Infasurf®) for

comparison with original observations (Survanta®). Unexpectedly, 10 μ M gramicidin did not cause the predicted Ca^{++} peaking within the time frame of the experiment (Figures 3A and B). Although gramicidin (open circles) induced a gradual rise in cytosolic $[\text{Ca}^{++}]$ observable only after 250 seconds (data not shown), the rise did not mimic the Ca^{++} peaking caused by PS+SAP (open and closed triangles). Moreover, the maximal $[\text{Ca}^{++}]$ (approximately 80 nM) associated with gramicidin exposure did not occur for greater than 400 seconds.

The failure of gramicidin — a molecule with hydrophobic properties similar to SP-B and SP-C — to mimic the effects of PS+SAP prompted us to reconstitute gramicidin with phospholipids. Reconstitution of gramicidin as a phospholipid mixture increased neutrophil $[\text{Ca}^{++}]$ mimicking the effect of PS+SAP in both magnitude and kinetics. 1% PLG (closed circles, Figures 3A and B) caused Ca^{++} peaking within 25 seconds followed by return to baseline levels similar to the patterns of both PS+SAP preparations. Also like the PS+SAP preparation, Survanta®, PLG induced Ca^{++} response in a logarithmic, dose-dependent manner (magnitude of Ca^{++} peak = $34.07 + 8.98 \{\ln[\text{PLG}]\}$; $R^2 = 0.88$) (data not shown).

The presence of extracellular Ca^{++} had limited demonstrable effect on results (Figure 3A versus 3B). Although Ca^{++} peaks induced by PS+SAP in the absence of extracellular Ca^{++} were higher [Infasurf® (open triangles): 182 ± 10 nM vs 144 ± 17 nM, $p < 0.05$; Survanta® (closed triangles): 131 ± 10 nM vs 90 ± 14 nM, $p < 0.05$] (Figures 3A and B), peaks were narrower and Ca^{++} runoffs were quicker. Presence of extracellular Ca^{++} had no demonstrable effect on PLG results [PLG (closed circles): 104 ± 13 nM vs 112 ± 15 nM, NS]. Among PS+SAP preparations, 1% Infasurf® induced consistently larger Ca^{++} responses than 1% Survanta® in both the presence and absence of extracellular Ca^{++} as quantified by either Ca^{++} peak data (see above) or by area under the curve data [extracellular Ca^{++} present: 141 ± 23 nM•min vs 32 ± 4 nM•min, $p < 0.01$ (Infasurf® vs Survanta®); extracellular Ca^{++} absent: 117 ± 13 nM•min vs 159 ± 20 nM•min, $p < 0.05$].

PS+SAP, PLG and gramicidin independently depolarize neutrophils. In an attempt to explain the observed differences in PLG and gramicidin effects on neutrophil Ca^{++} responses, magnitudes of depolarization induced by PLG and gramicidin were compared to that induced by PS+SAP preparation, Survanta[®]. Neutrophils were exposed (4 minutes, 25°C) to PS+SAP, PLG, gramicidin or control buffer, washed, and then resuspended in buffer. To measure membrane potential, suspended neutrophils were further diluted with buffer containing potentiometric dye, diOC₅(3) (225 nM final) in the spectrofluorometer. Upon stabilization of fluorescence, depolarization of neutrophils was completed with 150 mM KCl, and the change in fluorescent signal following prior PS+SAP, PLG or gramicidin exposure was expressed relative to fluorescent change observed under control conditions. These experiments demonstrated that prior exposure of neutrophils to 1% PS+SAP, 1% PLG or 10 μM gramicidin each independently decreased further membrane depolarization by 150 mM KCl $36\pm7\%$, $31\pm6\%$ and $34\pm2\%$, respectively. These results confirmed preexisting, partial membrane depolarization of approximately 25 mV (9) by 1% PS+SAP, 1% PLG and 10 μM gramicidin without significant differences among the three agents. Hence, differences in the Ca^{++} responses induced by gramicidin and PLG could not be explained by differing degrees of depolarization.

Inhibition of internal Ca^{++} store release eliminates Ca^{++} responses induced by PS+SAP and PLG. Prior experiments conducted in the absence of extracellular Ca^{++} strongly suggested that release of internal Ca^{++} storage sites provides the source of Ca^{++} responses. To distinguish between release of internal Ca^{++} stores and altered intracellular Ca^{++} buffering or extracellular Ca^{++} influx, direct inhibitors of Ca^{++} release from endoplasmic reticulum storage sites were employed. 2-APB inhibits Ca^{++} release by blocking G protein-sensitive, IP₃ receptors, and SKF-96365 inhibits both store-operated and receptor-mediated Ca^{++} release. Influences of inhibitors upon the PLG-induced responses were compared with those by fMLP and both PS+SAP preparations by comparing calculated areas under the time course curves of Ca^{++} responses. In

the presence of 2-APB at 30 or 100 μM or SKF-96365 at 10 μM concentrations, both inhibitors nearly eliminated neutrophil Ca^{++} responses to PLG, Survanta[®] and Infasurf[®] exposures (Figures 4A and B). By contrast, Ca^{++} responses to fMLP , a receptor-mediated process, were significantly decreased, but not eliminated. Though presented data come from experiments conducted in the presence of extracellular Ca^{++} , results in the absence of extracellular Ca^{++} were nearly identical (data not shown).

Inhibition of G protein activation limits Ca^{++} response induced by PS+SAP and PLG.

Inhibition of internal Ca^{++} release from storage sites influenced by G protein-coupled IP3 receptors suggested that upstream inhibition of G protein activation also would limit Ca^{++} response to PS+SAP and PLG. Accordingly, Ca^{++} responses to PLG, Survanta[®], Infasurf[®] and fMLP were compared with and without prior exposure to pertussis toxin. Preexposure to pertussis toxin reduced observed Ca^{++} responses in neutrophils by 95%, 74% and 48% by PLG, Survanta, and Infasurf, respectively (Figure 5). By contrast, at the fMLP dose employed, the response to fMLP was not significantly affected.

PS+SAP and PLG limit Ca^{++} influx by subsequent fMLP activation. Previously reported experiments by our laboratory demonstrated that preexposure of neutrophils to PS+SAP decreases subsequent Ca^{++} influx by fMLP activation of neutrophils (9). To compare effects of PLG with those of PS+SAP on subsequent activation of neutrophils, we examined the effect of PLG on Ca^{++} influx following fMLP stimulation. Neutrophils exposed to 1% PS+SAP, 1% PLG or control buffer were washed, suspended in Ca^{++} -free buffer, and then stimulated with 1 μM fMLP . After neutrophil $[\text{Ca}^{++}]$ peaked from fMLP -induced release of internal Ca^{++} stores, 10 mM CaCl_2 was added to the extracellular space. Influx of calcium was measured over 2 minutes, and net Ca^{++} responses were compared by calculating areas under the curves of falling neutrophil $[\text{Ca}^{++}]$ (9). fMLP -stimulated Ca^{++} influx was reduced significantly relative to control by

pretreatment of neutrophils with either PS+SAP or PLG [control (MLP only): 204 ± 22 nM•min; PS+SAP: 95 ± 31 nM•min, and PLG: 131 ± 31 nM•min; $p < 0.01$ (PS+SAP or PLG vs control)].

DISCUSSION

Commercially available natural surfactant (PS+SAP) significantly improves survival in adult respiratory distress syndrome (17,18). In neonatal respiratory distress syndrome, PS+SAP promotes better short and long-term outcomes than PS-SAP (3). These clinical investigations demonstrating therapeutic superiority of PS+SAP over PS-SAP suggest that presence of surfactant-associated apoproteins, SP-B and SP-C, account for the improved clinical outcomes.

Neutrophils are the primary cellular mediators of acute inflammation and are largely responsible for the lung damage seen in respiratory distress syndromes. During development of adult respiratory distress syndrome, neutrophils migrate into lung parenchyma whereupon releasing proteases and active oxygen radicals, they injure the respiratory epithelium and inactivate native pulmonary surfactant (19). Accordingly, investigators have examined the possibility that improved clinical benefits realized with PS+SAP treatment might be the result of suppressed neutrophil functions. Suwabe *et al.* demonstrated that Surfacten[®], a PS+SAP preparation, inhibits adherence and superoxide production by human neutrophils (5). Ahuja *et al.* reported that native porcine surfactant, but not dipalmitoylphosphatidylcholine preparation, inhibits human neutrophil superoxide production induced by either fMLP or phorbol myristate acetate (6). Tegtmeier *et al.* observed that elastase release by activated human neutrophils is decreased by natural surfactants, Survanta[®] and Curosurf[®], whilst synthetic surfactant, Exosurf[®], exhibits only modest effects (8). We have recently reported that Survanta[®] decreases neutrophil adherence and aggregation following physiologic stimulation while Exosurf[®] does not (9). The pathway by which these inhibitory effects upon neutrophils occur is the focus of our present experiments.

A common link among most neutrophil functions is change in cytosolic Ca^{++} activity. An increase in cytosolic $[\text{Ca}^{++}]$ is the earliest measurable event following neutrophil

activation, and it serves to initiate or enhance response (20,21). Therefore, during our earliest investigations, it was tempting to propose that PS+SAP inhibits neutrophil function via direct modulation of neutrophil $[Ca^{++}]$. It was established by those earlier investigations of activated neutrophils that PS+SAP does inhibit Ca^{++} influx into neutrophils activated by fMLP and other physiologic stimulants (9). However, it remained unknown how PS+SAP inhibits Ca^{++} influx or whether PS+SAP directly releases internal Ca^{++} stores. The goal of present experiments was to address the latter question regarding direct influences of PS+SAP and of gramicidin, a related channel-forming agent, on release of internal Ca^{++} stores.

By these experiments, we first established that two different preparations of PS+SAP induce transient peaking of cytosolic Ca^{++} via the release of internal Ca^{++} stores (Figures 1 and 3). By contrast, neither PS-SAP nor purified phospholipids (PL) induced transient Ca^{++} peaking. Though a possible contribution of extracellular Ca^{++} to the observed responses was not excluded, the similarity of responses in the presence or absence of extracellular Ca^{++} suggests that the extracellular contribution is minimal. Evidence for a causal relationship between PS+SAP and internal Ca^{++} release was demonstrated by: 1) consistent stimulus-response timing between presentation of PS+SAP and peaking of cytosolic $[Ca^{++}]$; 2) a dose-response relationship between $[PS+SAP]$ and magnitude of Ca^{++} peaking; 3) elimination of response by specific internal Ca^{++} release inhibitors (2-APB and SKF-96365); 4) lack of an observed response in the absence of SAP; and 5) plausibility of cation channel insertion in neutrophil plasma membrane as the initiating mechanism (10). Differences in the magnitudes of Ca^{++} responses between the two 1% PS+SAP preparations (Figures 3A and B) is likely related to significantly higher SAP concentrations in Infasurf[®] and/or the relative lack of SAP-B in Survanta[®] (22). Duplication of these results by the exposure of neutrophils to PLG — a well-characterized, cation channel protein packaged in phospholipids resembling PS — corroborates the argument for cation channel insertion as the initiating event.

Gramicidin is a small, hydrophobic peptide that has been extensively studied for its ability to form monovalent cation channels in cell membranes. It exists naturally in two basic conformations – a double helix and a β -helical dimer (14,23). The latter conformation is the one believed responsible for forming channels in cell membranes (14,24). Surfactant-associated apoproteins SAP-B and SAP-C are hydrophobic lipopolypeptides containing extended α -helical regions within their secondary structures (1). Amphipathic, α -helical structure within the peptide subunits resemble other monomeric proteins recognized for insertion in membrane bilayers and for combination with other subunits forming channel oligomers (25). In this regard, the SP-C α -helix is 37Å long with a central hydrophobic region 23Å long (26). This structure provides sufficient length to span the hydrophobic domain of plasma membrane bilayers (27). It has been observed that upon interaction with bilayers, the SP-C molecule inserts by spanning the bilayer in an α -helical configuration (28). Coupled with the observation that the SP-C helix spans bilayers with a 24° tilt (29), SP-C appears well suited to comprise part of an oligomeric channel complex (28). By contrast, its propensity to exist in monomeric state within lipid bilayer (30) limits its suitability for channel formation in pure form. In this regard, SP-B might complex with SP-C to form a channel oligomer. The structure of SP-B is homologous with that of other saposin-like proteins including the *Entamoeba histolytica* pore-forming peptide (31). SP-B is larger than SP-C with approximately half of the molecule in α -helical configuration. However, in contrast to the transmembrane orientation of SP-C in lipid bilayer, SP-B interacts with bilayers by shallow anchoring at the membrane surface (32), thus aligning parallel with the bilayer axis. It may prove significant that the 39pS conductance of Survanta®, observed in our laboratory (10), resembles the 40pS conductance associated with pentameric clusters of α -helices forming other cation channels in lipid bilayers (33).

Insertion of monovalent, cation channels by PS+SAP and PLG would be expected to depolarize neutrophils in the K⁺-predominant intracellular and Na⁺-predominant extracellular

environments. Consistent with this expectation, current experiments also established that PS+SAP (and PLG) depolarize neutrophils and that prior polarization of neutrophils is necessary for the Ca^{++} peaking. This was substantiated by the observed, semi-quantitative membrane depolarization following PS+SAP or PLG exposure and by the dependence of Ca^{++} response upon preexisting neutrophil polarization (Figure 2). Substitution of N-methylglucamine for NaCl also demonstrated dependency of Ca^{++} peaking upon extracellular Na^+ presence. This Na^+ dependency is best explained by: 1) the demonstrated Na^+ conductances of SAP and gramicidin channels inserted in plasma membrane (10); and 2) elimination of available monovalent cations for channel conduction following removal of extracellular Na^+ . Put another way, N-methylglucamine hyperpolarizes and fixes membrane potential by eliminating extracellular cations capable of carrying charge. However, despite the demonstrated necessity for preexisting polarization and subsequent depolarization after exposure, these experiments did not prove that depolarization by SAP or PLG channel insertion is sufficient for release of internal Ca^{++} stores. Our observations that both PLG and gramicidin caused equally effective membrane depolarization in the absence of Ca^{++} peaking by gramicidin (Figures 3A and 3B) and that KCl-induced depolarization did not cause $[\text{Ca}^{++}]$ elevation (Figure 2A, open circles) confirm that membrane depolarization alone is not sufficient. Analogous to this, ΔMLP is known to both depolarize neutrophil membranes and release internal Ca^{++} stores sensitive to membrane potential (34), but depolarization is not considered sufficient for the receptor-mediated release of internal Ca^{++} (35).

To explain the potentiometric sensitivity of the PS+SAP-induced Ca^{++} response, we speculated that internal Ca^{++} release is related to membrane potential through an unidentified intermediate event coupling membrane potential change by PS+SAP to internal Ca^{++} release. This possibility was supported by observed 2-APB inhibition in our experiments that is known to inhibit G protein-activated Ca^{++} store release in neutrophils (36). It was strengthened by

observations that pore-forming toxins induce membrane depolarization accompanied by G protein-activated Ca^{++} release from internal stores in neutrophils (16). In pursuit of this possibility, the contribution of pertussis toxin-sensitive G protein activation to Ca^{++} release was explored. We found that pertussis toxin fully inhibited PLG-induced Ca^{++} release and partially inhibited PS+SAP-induced release 48-74%. Closer review of the time courses for PS+SAP-induced releases revealed that much of the pertussis toxin-dependent inhibition occurred shortly after PS+SAP exposure thereby delaying the observed Ca^{++} peaks approximately 60 seconds and reducing magnitudes (data not shown). The delay in Ca^{++} peaks suggested that at least two pathways — one dependent and one independent of pertussis toxin-sensitive, G protein activation — contribute to Ca^{++} release.

Present experiments do not explain how preexposure of neutrophils to PS+SAP or PLG inhibits subsequent Ca^{++} influx during activation by fMLP . However, the common sensitivities to 2-APB and SKF-96365 of PS+SAP-, PLG- and fMLP -induced Ca^{++} responses suggest that depletion of internal Ca^{++} stores is an intermediate event coupling PS+SAP or PLG preexposure with inhibition of fMLP -induced Ca^{++} influx.

In summary, our experiments demonstrate that both PS+SAP and PLG preparations independently depolarize neutrophils, release Ca^{++} from internal stores by a G protein-activated pathway, and alter subsequent neutrophil responses to recognized physiologic stimulants. We speculate that both PLG and PS+SAP insert monovalent cation channels into neutrophil membranes causing cell depolarization and G protein-mediated release of intracellular Ca^{++} stores. This PS+SAP-initiated cascade of events modulates the response of human neutrophils during inflammation to improve clinical outcomes in patients with respiratory distress syndromes. Although the PS+SAP preparation currently employed does not contain the other two major surfactant apoproteins, both SAP-A and SAP-D are believed to play important roles in local lung defense by enhancing chemotaxis, opsonizing bacteria, enhancing macrophage

function and stimulating antibody production (37,38). Therefore, it would appear that in addition to reducing surface tension, native pulmonary surfactant shares both pro- and anti-inflammatory properties. For this reason it is enticing to speculate that in the course of pulmonary disease a balance is sought between the two immunomodulatory arms of surfactant, whether it be from invading microorganisms or an overexuberant host inflammatory response.

REFERENCES

FIGURE LEGENDS

FIGURE 1. Effect of PS+SAP on neutrophil $[Ca^{++}]$ response. Neutrophils suspended in Ca^{++} -free buffer were exposed to 1% PS+SAP (closed circles), 1% PS-SAP (open circles) or 1% purified phospholipids (closed triangles) at time 0. $[Ca^{++}]$ response was monitored over time. Data are expressed as mean \pm SE values (n=4).

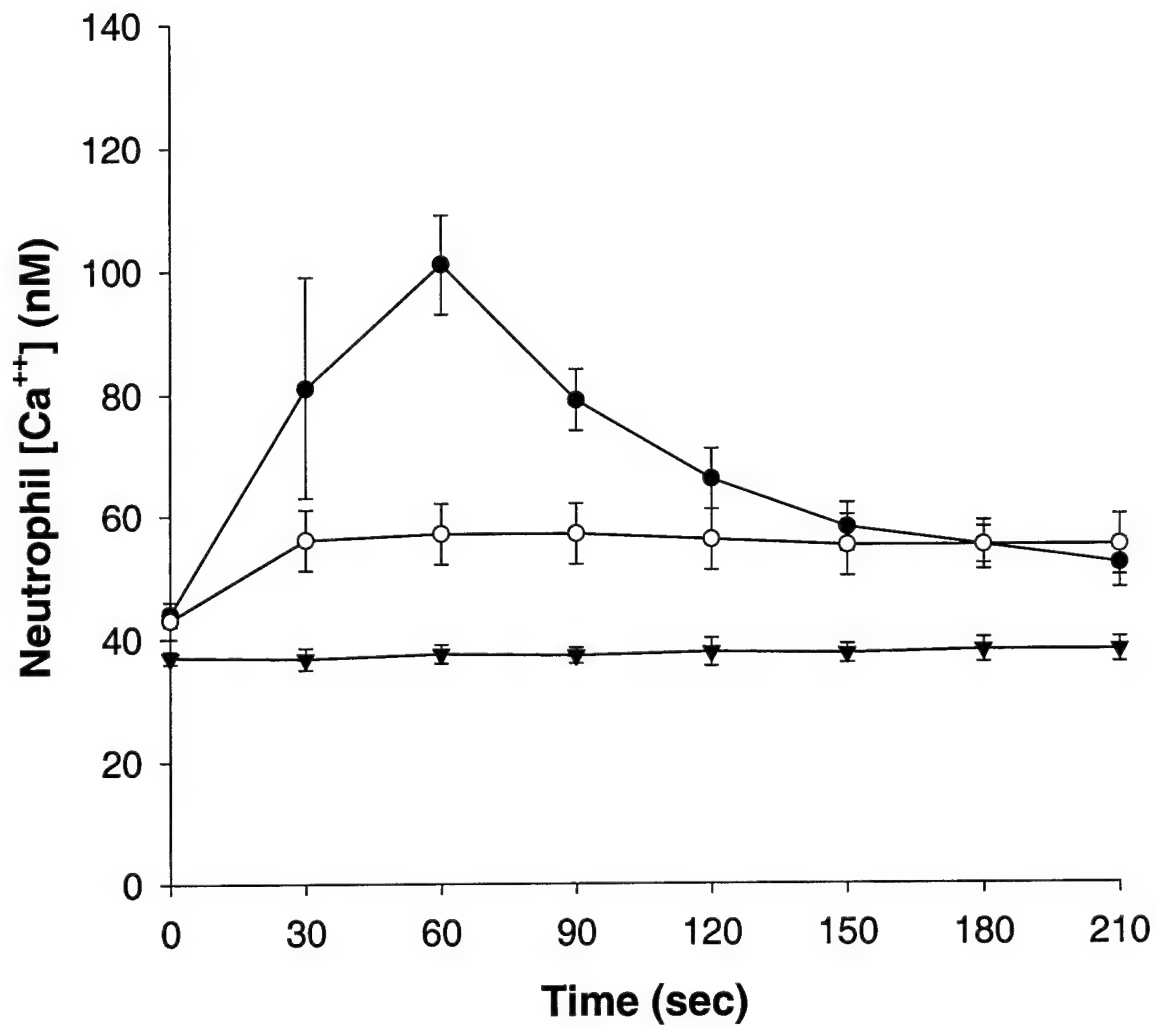


FIGURE 2. Inhibition of PS+SAP-induced $[Ca^{++}]$ response by neutrophil depolarization or extracellular Na^+ removal.

Panel A: Prior to exposure of neutrophils to 1% PS+SAP at time 0, neutrophils were suspended in either Ca^{++} -free KCl buffer (open circles) or Ca^{++} -free NaCl buffer containing 90 μ M gramicidin (closed triangles) to depolarize neutrophils, or in Ca^{++} -free NaCl buffer (control, closed circles). $[Ca^{++}]$ response was monitored over time. Data are expressed as mean \pm SE values (n=4).

Panel B: Prior to exposure of neutrophils to 1% PS+SAP at time 0, neutrophils were suspended in either Ca^{++} -free NaCl buffer (control, closed circles) or Ca^{++} -free buffer substituting N-methylglucamine for NaCl (open circles). $[Ca^{++}]$ response was monitored over time. Data are expressed as mean \pm SE values (n=4).

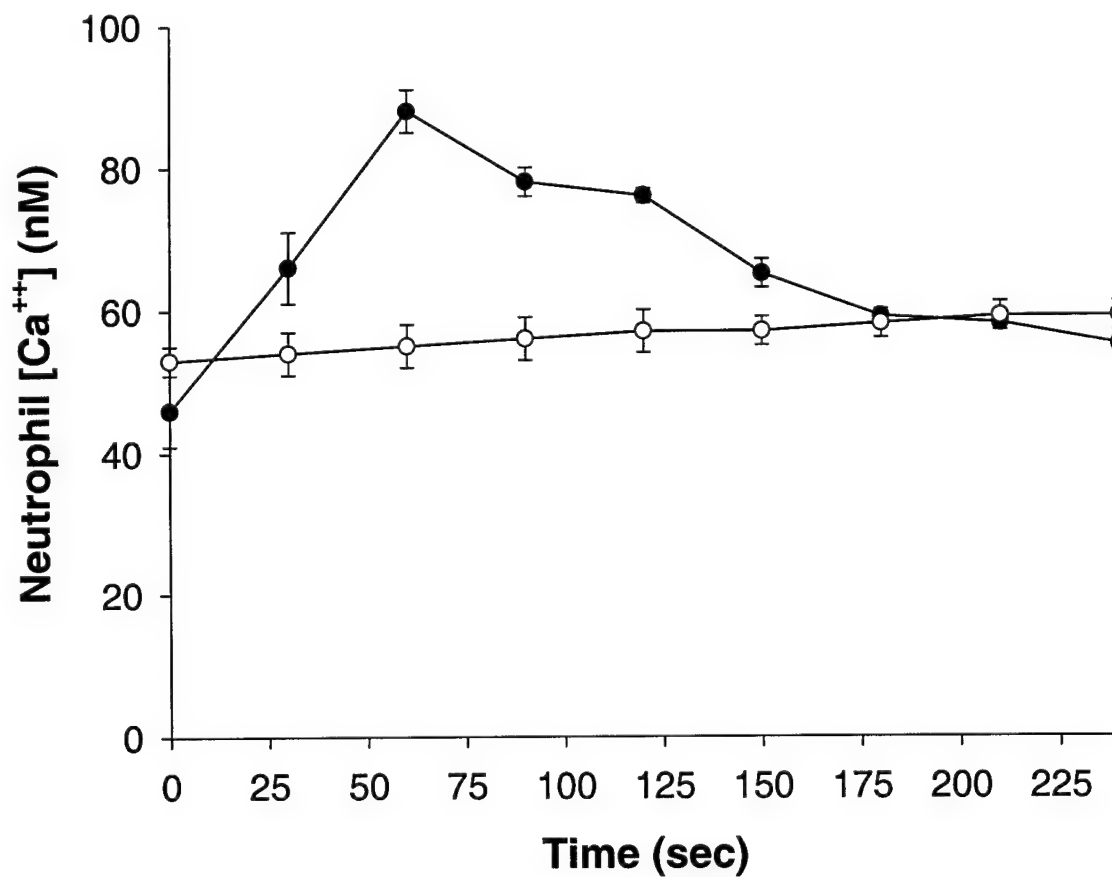
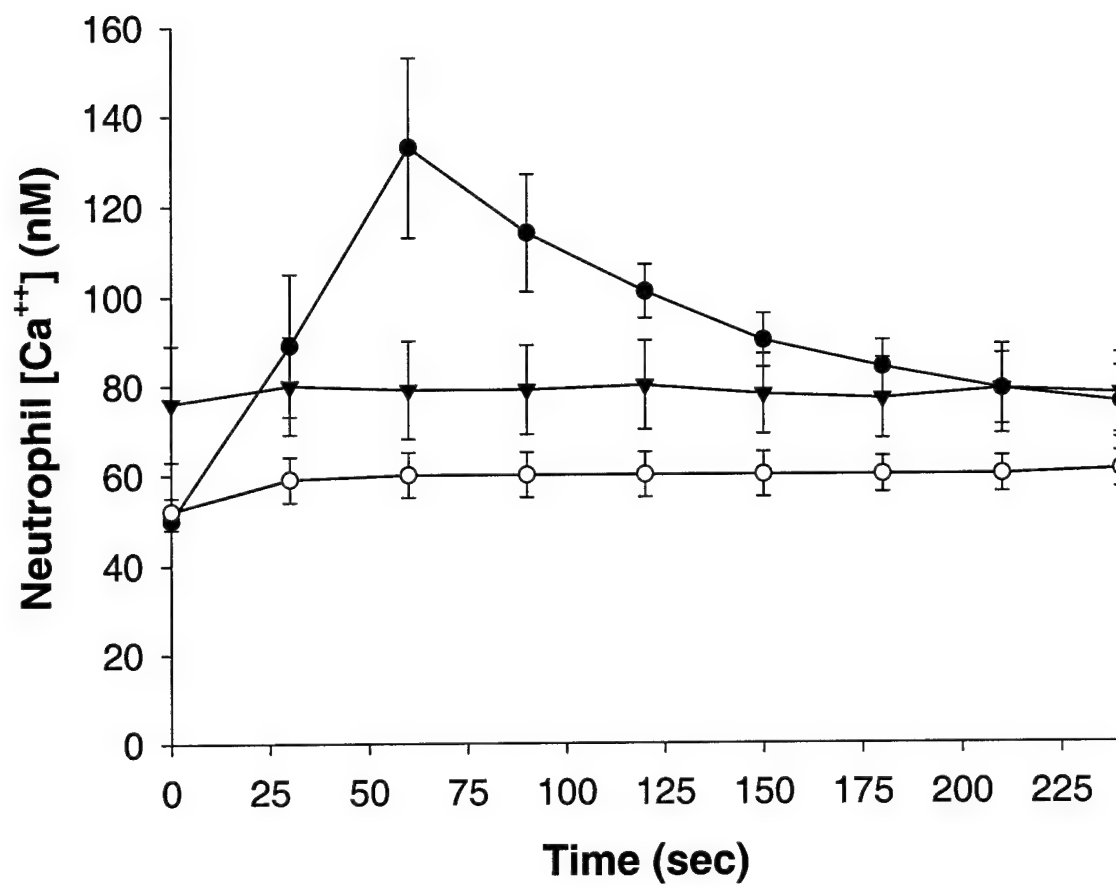


FIGURE 3. Effect of PS+SAP, PLG or gramicidin on neutrophil $[Ca^{++}]$ response.

Neutrophils suspended in Ca^{++} -supplemented (Panel A) or Ca^{++} -free (Panel B) buffer were exposed to either of two 1% PS+SAP preparations (closed triangles (Survanta[®]) or open triangles (Infasurf[®])), 1% PLG (closed circles) or 10 μ M gramicidin (open circles) at time 0. $[Ca^{++}]$ response was monitored over time. Data are expressed as mean \pm SE values (n=5-8).

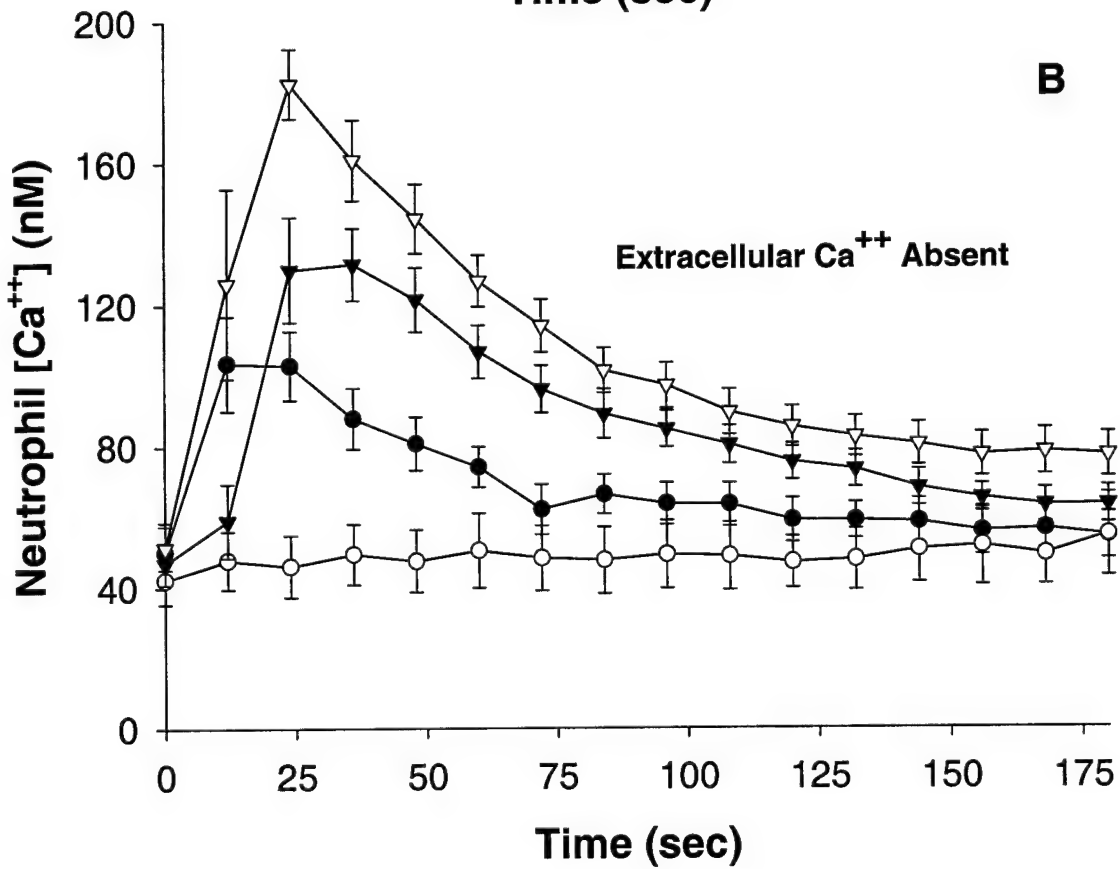
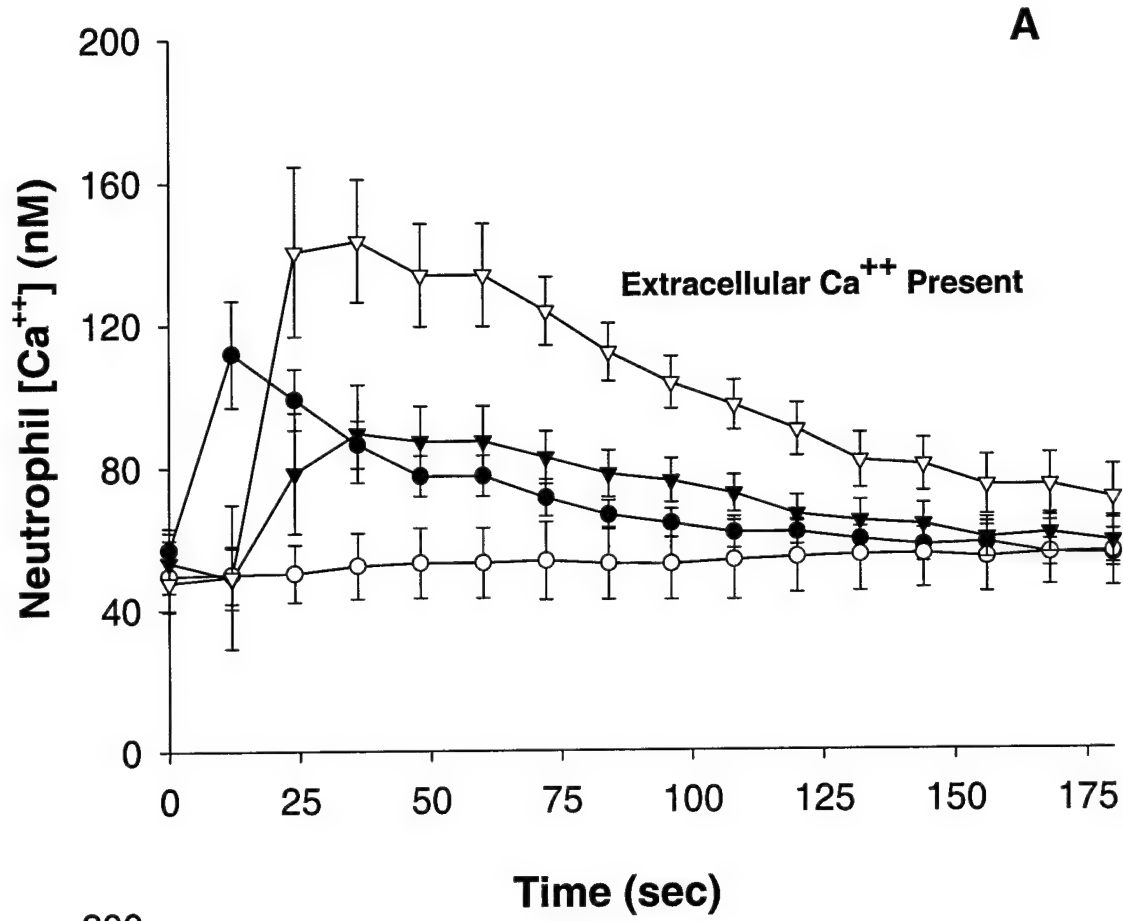


FIGURE 4. Effect of Ca^{++} store release inhibitors on PLG-, PS+SAP- or fMLP-induced Ca^{++} responses. Two Ca^{++} store release inhibitors were employed — 2-APB at 30 or 100 μM (Panel A) or SKF-96365 at 10 μM (Panel B). Inhibitor or equal volume of solvent was added to neutrophils suspended in Ca^{++} -supplemented buffer 4 minutes prior to addition of 1% PLG, 1% PS+SAP (Survanta[®] or Infasurf[®]), or 1 μM fMLP. Ca^{++} responses were monitored and data are presented as areas under the Ca^{++} response curve for the first 240 seconds of exposure (n=4-6).

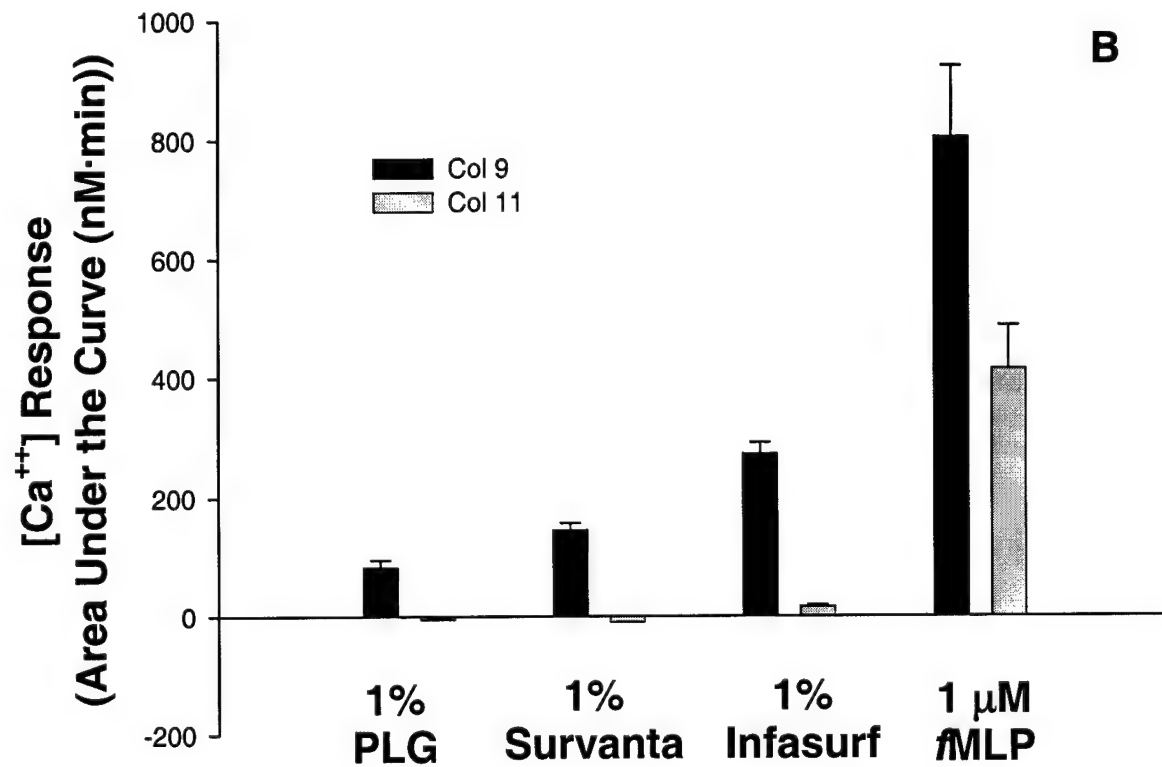
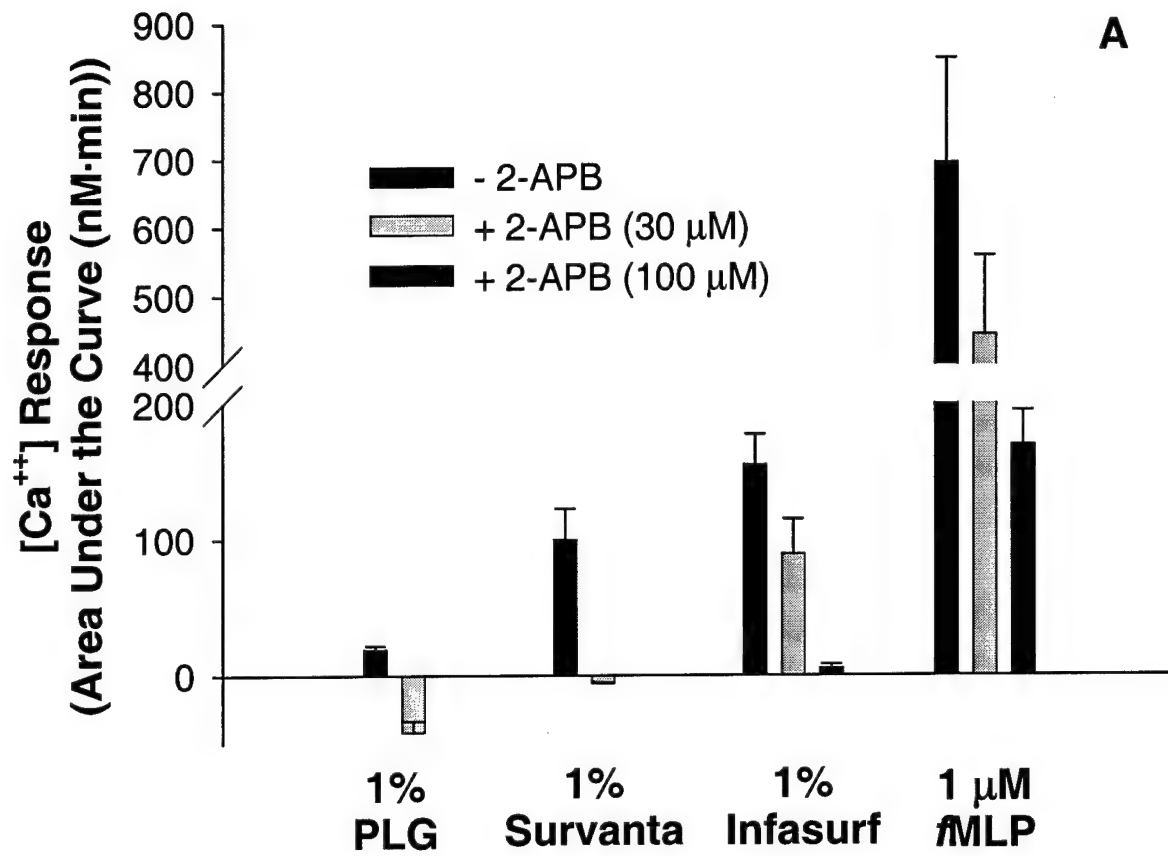
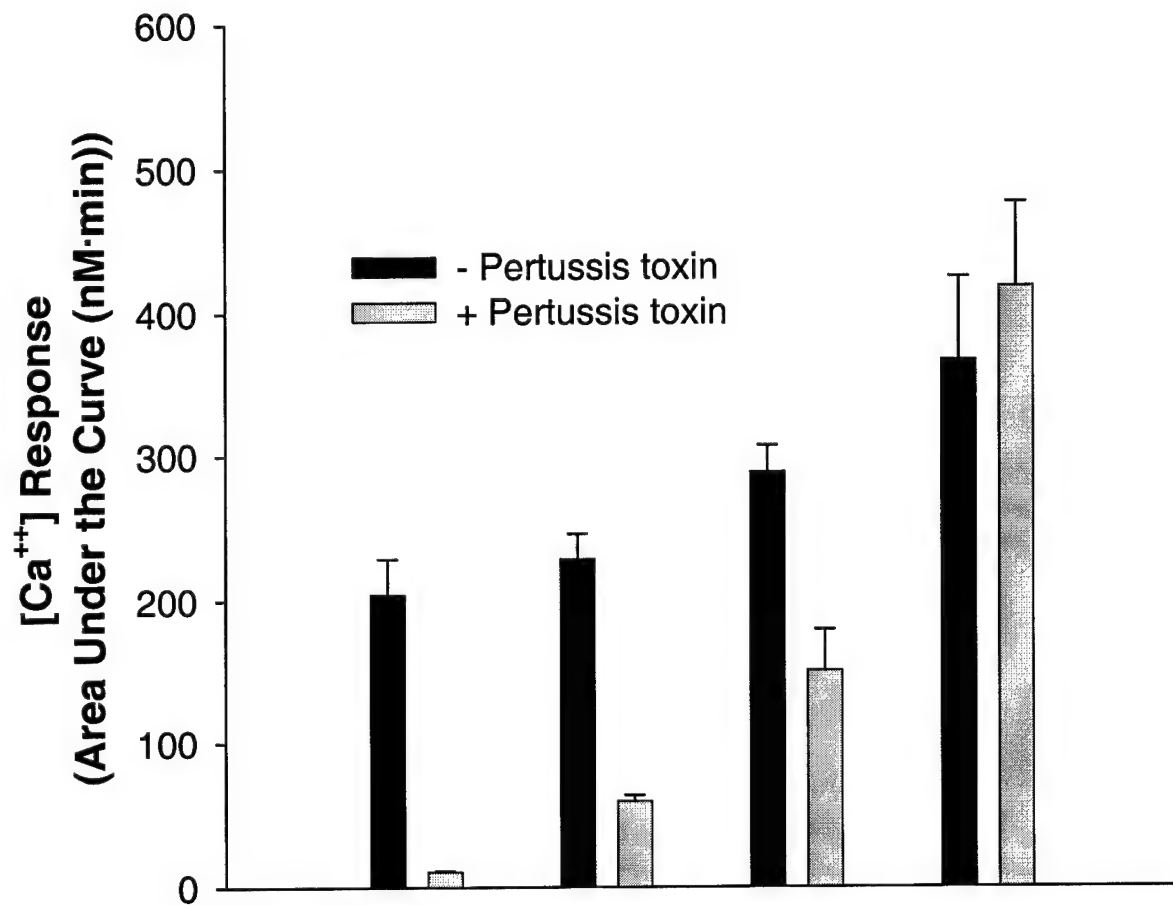


FIGURE 5. Effect of G protein activation inhibitor on PLG-, PS+SAP- or fMLP-induced Ca^{++} responses. Neutrophils suspended in Ca^{++} -supplemented buffer were exposed to pertussis toxin or solvent as described in text before washing, loading with fura2, and exposing to 1% PLG, 1% PS+SAP (Survanta® or Infasurf®), or 1 μM fMLP. Ca^{++} responses were monitored and data are presented as areas under the curve as explained for Figure 4 (n=4).



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- 1 . Hawgood S 1991 Structures and properties of the surfactant-associated proteins. *Ann Rev Physiol* 53:375-94
 - 2 . Kattwinkel J 1998 Surfactant-evolving issues. *Clin Perinatol* 25:17-31
 - 3 . Halliday HL 1996 Controversies: Synthetic or natural surfactant. The case for natural surfactant. *J Perinat Med* 24:417-26
 - 4 . Henson PM, Johnston BR 1987 Tissue injury in inflammation: Oxidants, proteases, and cationic proteins. *J Clin Invest* 79:669-674
 - 5 . Suwabe A, Otake K, Yakuwa N, Suzuki H, Ito M, Tomoike H, Saito Y, Takahashi K 1998 Artificial surfactant (Surfactant TA) modulates adherence and superoxide production of neutrophils. *Am J Respir Crit Care Med* 158:1890-9
 - 6 . Ahuja A, Oh N, Chao W, Spragg RG, Smith RM 1996 Inhibition of the human neutrophil respiratory burst by native and synthetic surfactant. *Am J Respir Cell Mol Biol* 14:496-503
 - 7 . Finck CM, Hodell MG, Marx WH, Paskanik AM, McGraw DJ, Lutz CJ, Gatto LA, Picone AL, Nieman GF 1998 Endotoxin-stimulated alveolar macrophage recruitment of neutrophils and modulation with exogenous surfactant. *Crit Care Med* 26:1414-8
 - 8 . Tegtmeier FK, Gortner L, Ludwig A, Brandt E 1996 In vitro modulation of induced neutrophil activation by different surfactant preparations. *Eur Respir J* 9:752-7
 - 9 . Chacon-Cruz E, Buescher ES, Oelberg DG 2000 Surfactant modulates the calcium response of neutrophils to physiologic stimulation via cell membrane depolarization. *Pediatr Res* 47:405-413
 - 10 . Oelberg DG, Xu F 2000 Pulmonary surfactant proteins insert cation-permeable channels in planar bilayers. *Mol Genet Metab* 70:295-300

-
- 11 . Boyum A 1968 Isolation of mononuclear cells and granulocytes from human blood.
Scand J Clin Lab Invest 97:77-89
 - 12 . Yamada T, Ikegami M and Jobe AH 1990 Effects of surfactant subfractions on preterm rabbit lung function. Pediatr Res 27:592-8
 - 13 . Killian JA 1992 Gramicidin and gramicidin-lipid interactions. Biochim Biophys Acta 1113:391-425
 - 14 . Hansen CA, Monck JR, Williamsons JR 1990 Measurement of intracellular free calcium to investigate receptor-mediated calcium signaling. Methods Enzymol 191:691-706
 - 15 . Hauser CJ, Fekete Z, Adams JM, Garced M, Livingston Dh, Deitch EA 2001 PAF-mediated Ca^{2+} influx in human neutrophils occurs via store-operated mechanisms J Leukoc Biol 69:63-68
 - 16 . Krause KH, Fivaz M, Monod A, van der Goot FG 1998 *Aerolysin* induces G-protein activation and Ca^{++} release from intracellular stores in human granulocytes. J Biol Chem 273:18122-18129
 - 17 . Richman PS, Spragg RS, Robertson B, Merritt TA, Curstebt T 1989 The adult respiratory distress syndrome: first trials with surfactant replacement. Eur Respir J 2:109-11
 - 18 . Enhorning G 1989 Surfactant replacement in adult respiratory distress syndrome. Am Rev Respir Dis 140:281-3
 - 19 . Tate RM, Repine JE 1983 Neutrophils and the adult respiratory distress syndrome. Am Rev Respir Dis 128:552-9
 - 20 . Korchak HM, Vienne K, Rutherford LE, Wilkenfeld C, Finkelstein MC, Weissman G 1984 Stimulus response coupling in the human neutrophil. II. Temporal analysis of changes in cytosolic calcium and calcium efflux. J Biol Chem 259:4076-4082

-
- 21 . Von Tscharner V, Prod'hom B, Baggiolini M, Reuter H 1986 Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature* 324:369-72
 - 22 . Notter RH. *Lung Surfactants: Basic Science and Clinical Applications*. New York: Marcel Dekker, 2000, pp 322-324.
 - 23 . Anderson OS 1984 Gramicidin channels. *Ann Rev Physiol* 46:531-48
 - 24 . Kovacs F, Quine J, Cross TA 1999 Validation of the single-stranded channel conformation of gramicidin A by solid-state NMR. *Proc Natl Acad Sci USA* 96:7910-5
 - 25 . Engelman DM 1996 Crossing the hydrophobic barrier: Insertion of membrane proteins *Science*, 274:1850-51
 - 26 . Johansson J, Szyperski T, Curstedt T and Wuthrich K, 1994 The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich α -helix *Biochemistry* 33:6015-23
 - 27 . Montal M 1990 Molecular anatomy and molecular design of channel proteins *FASEB J* 4:2623-35
 - 28 . Vandenbussche G, Clercx A, Curstedt T, Johansson J, Jornvall H and Ruyschaert JM 1992 Structure and orientation of the surfactant-associated protein C in a lipid bilayer *Eur J Biochem* 203:201-9
 - 29 . Pastrana B, Mautone AJ and Mendelsohn R 1991 Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids *Biochemistry* 30:10058-64
 - 30 . Horowitz AD, Baatz JE and Whitsett JA 1993 Lipid effects on aggregation of pulmonary surfactant protein SP-C studied by fluorescence energy transfer *Biochemistry*, 32:9513-23

-
- 31 . Vaccaro AM, Salvioli R, Tatti M and Ciaffoni F 1999 Saposins and their interaction with lipids *Neurochem. Res.*, 24:307-14
 - 32 . Vandebussche G, Clercx A, Clercx M, Curstedt T, Johansson J, Jornvall H and Ruyschaert JM 1992 Secondary structure and orientation of the surfactant protein SP-B in a lipid environment. A Fourier transform infrared spectroscopy study *Biochemistry* 31:9169-76
 - 33 . Grove A, Iwamoto T, Montal MS, Tomich JM and Montal M 1992 Synthetic peptides and proteins as models for pore-forming structure of channel proteins *Methods Enzymol.*, 207:510-25
 - 34 . Di Virgilio F, Lew PD, Andersson T and Pozzan T. 1987 Plasma membrane potential modulates chemotactic peptide-stimulated cytosolic free Ca^{2+} changes in human neutrophils *J Biol Chem* 262:4574-4579
 - 35 . Andersson T, Dahlgren C, Pozzan T, Stendahl O and Lew PD 1986 Characterization of fMet-Leu-Phe receptor-mediated Ca^{2+} influx across the plasma membrane of human neutrophils *Molecular Pharmacol* 30:437-443
 - 36 . Maruyama T, Kanaji T, Nakade S, Kanno T and Mikoshiba K 1997 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release *J Biochem* 122:498-505
 - 37 . Wright JR 1997 Immunomodulatory functions of surfactant. *Physiol Rev* 77:931-62
 - 38 . Cai GZ, Griffin GL, Senior RM, Longmore WJ, Moxley MA 1999 Recombinant SP-D carbohydrate recognition domain is a chemoattractant for human neutrophils. *Am J Physiol* 276:L131-6